J Biol Inorg Chem (2013) 18:865–866
DOI 10.1007/s00775-013-1020-6

COMMENTARY

Cytochrome *c* and superoxide

Willem H. Koppenol

Received: 6 May 2013 / Accepted: 20 June 2013 / Published online: 18 July 2013

Abstract Wegerich et al. (*J. Biol. Inorg. Chem.* 18:429–440, 2013), working with singly modified human cytochromes *c*, claim to have found a new mechanism for the reduction of iron(III) cytochrome *c* by superoxide. I show that electron transfer by way of the solvent-accessible haem edge—a mechanism not considered by Wegerich et al.—is still the correct mechanism. Furthermore, several deficiencies in this work preclude any comparisons with other publications on this topic.

Recently, an article entitled "Mechanistic insights into the superoxide–cytochrome *c* reaction by lysine surface scanning" [1] was published in this journal. Wegerich et al. wrote that cytochrome *c* has been used for a long time to monitor the reactions of superoxide, which is correct, and that "little is known about the mechanism of the interaction of the small charged radical and the non-uniformly charged redox protein", which is not. According to Wegerich et al. [1], the possibilities for reduction are long-range electron transfer from a positive surface charge to the haem, or the superoxide entering the haem cavity of cytochrome *c*. The well-established mechanism of reaction at the solvent-accessible haem edge [2–4], based on reactions of singly modified cytochromes *c* [5–9], is not mentioned. As superoxide does not react with amino acids [10], reduction by way of the solvent-accessible haem edge is, in fact, the only possible mechanism. In the case of negatively charged reactants, including superoxide [11], reaction at that location is favoured by the evolutionarily conserved [12] asymmetric electric potential field of cytochrome *c* [13]. The left side of Fig. 3 in Wegerich et al. [1] shows a beautiful view of the front of cytochrome *c* down the dipole vector, the positive end of which emerges near Phe82, close to the solvent-accessible haem edge. Regrettably, the source of this figure is not the reference indicated in the legend.

The mechanism explored by the authors goes back ultimately to a hypothesis of Winfield [14] that aromatic amino acids could be stepping stones during electron transfer. Dickerson applied this to cytochrome *c* and suggested that electron transfer occurs from the surface to the haem via Tyr74, Trp59 and Tyr67. It is this mechanism that Butler et al. [15] adopted for the reaction of superoxide and iron(III) cytochrome *c*. Later the Dickerson–Winfield mechanism was abandoned by Dickerson and Timkovich [16] because in cytochrome *c*-550 the position of Tyr74 is occupied by a leucine. This pathway for the electron was never meant to be a channel through which ions can reach the haem iron. Although anions such as cyanide can reach the haem iron, that reaction proceeds via the alkaline conformation of iron(III)cytochrome *c*, and is too slow—near neutral pH as well as at higher pH [17]—to be relevant to the reaction of iron(III) cytochrome *c* and superoxide. However, if the energy to create a tyrosyl radical is available, the Winfield mechanism does apply to electron transfer in, for instance, ribonucleotide reductase, where a tyrosyl radical is generated by a high oxidation state of a two-iron centre followed by electron "hopping" over 3.5 nm, assisted by three tyrosines and possibly a tryptophan [18]. Thus, Wegerich et al. have misinterpreted a mechanism that has been found unsuitable for cytochrome *c* for 38 years.

Wegerich et al. [1] show that they are not familiar with the cytochrome *c* literature. It is curious that they measured

W. H. Koppenol (✉)
Institute of Inorganic Chemistry, Department of Chemistry and Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland
e-mail: koppenol@inorg.chem.ethz.ch

the difference in the extinction coefficients of iron(III) cytochrome *c* and iron(II) cytochrome *c* at 550 nm to determine the concentration, but did not compare their difference of $24.6 \times 10^3\ M^{-1}\ cm^{-1}$ with that of Van Gelder and Slater [19] of $21.1 \times 10^3\ M^{-1}\ cm^{-1}$. Similarly, the electrode potential of iron(III) cytochrome *c*/iron(II)cytochrome *c* is well known, +260 mV versus the normal hydrogen electrode for horse heart cytochrome *c* [20], but the value given by Wegerich et al. [1], 15 mV for horse cytochrome *c*, is not compared with that value. The reader finds in the text of the "Results and discussion" section—not in the "Materials and methods" section and not in the legend of Table 1, which lists the electrode potentials of horse, human and many singly modified cytochromes *c*—that an Ag/AgCl reference electrode was used. This would add 197 mV (saturated KCl) to 250 mV (0.60 m KCl), but because Wegerich et al. do not divulge what the chloride concentration of the reference electrode is, one cannot know what the electrode potentials of these cytochromes *c* are. In addition, the indirectly determined rate constant of horse cytochrome *c* is not compared with that obtained, directly by pulse radiolysis, by Butler et al. [15] or with other such published values [11, 21, 22]. The rate constant is very ionic strength dependent, with an extrapolated value of $3 \times 10^6\ M^{-1}\ s^{-1}$ at $I = 0$ [22] and pH 7.1 and $2.6 \times 10^5\ M^{-1}\ s^{-1}$ at 50 mM phosphate, pH 7.8 [11]. The ionic strength of the indirect assay is not given, but the reported rate constant of $6.7 \times 10^4\ M^{-1}\ s^{-1}$ near neutral pH [1] is outside the realm of reasonable rate constants. Wegerich et al. [1] used xanthine oxidase, a large protein of 270 kDa, to generate superoxide, which either disproportionates or reacts with cytochrome *c*. As the rate constant of disproportionation as a function of pH is known precisely [23], the rate constant of the reaction with cytochrome *c* can be calculated from the amount of iron(II) cytochrome *c* produced. In 1973, Sawada and Yamazaki [24] used this method and found a rate constant that was approximately tenfold smaller than the rate constants found—later—in the pulse radiolysis studies cited above. Thus, this method to determine the rate constant of superoxide with iron(III) cytochrome *c* yields incorrect rate constants and should not have been used (see also [25]). Why this method does not yield reliable results is not clear. The production of superoxide by xanthine oxidase may be influenced by the binding of other substrates or the binding of inhibitors at sites remote from the xanthine binding site [26]. Furthermore, superoxide may react with the substrates and the products of the enzymatic reaction—hypoxanthine, xanthine or urate—although no rate constants could be found. Wegerich et al. [1] compared the rate constants of reduction by superoxide of singly modified human cytochromes *c* that have an added positive charge. Given that the method does not yield reliable values, one cannot interpret the results. Another concern is the possible presence of trace amounts of copper during the assay. We showed that such contamination influences the yields and kinetics of the reaction of cytochrome *c* with superoxide and advocated the use of the metal chelator edta [11, 22]. Given these concerns, we urge the authors to determine rate constants directly—by pulse radiolysis—and in the presence of edta.

Acknowledgments I thank R. Kissner and P.L. Bounds for helpful comments.

References

1. Wegerich F, Giachetti A, Allegrozzi M, Lisdat F, Turano P (2013) J Biol Inorg Chem 18:429–440
2. Ahmed A, Millett F (1981) J Biol Chem 256:1611–1615
3. Butler J, Davies DM, Sykes AG, Koppenol WH, Osheroff NO, Margoliash E (1981) J Am Chem Soc 103:469–471
4. Butler J, Chapman SK, Davies DM, Sykes AG, Speck SH, Osheroff NO, Margoliash E (1983) J Biol Chem 258:6400–6404
5. Brautigan DL, Ferguson-Miller S, Margoliash E (1978) J Biol Chem 253:130–139
6. Brautigan DL, Ferguson-Miller S, Tarr GE, Margoliash E (1978) J Biol Chem 253:140–148
7. Ferguson-Miller S, Brautigan DL, Margoliash E (1978) J Biol Chem 253:149–159
8. Osheroff NO, Brautigan DL, Margoliash E (1980) J Biol Chem 255:8245–8251
9. Smith MB, Stonehuerner J, Ahmed AQJ, Staudenmeyer N, Millett F (1980) Biochim Biophys Acta 592:303–313
10. Bielski BHJ, Cabelli DE, Arudi RL, Ross AB (1985) J Phys Chem Ref Data 14:1041–1100
11. Butler J, Koppenol WH, Margoliash E (1982) J Biol Chem 257:10747–10750
12. Koppenol WH, Rush JD, Mills JD, Margoliash E (1991) Mol Biol Evol 8:545–558
13. Koppenol WH, Margoliash E (1982) J Biol Chem 257:4426–4437
14. Winfield ME (1965) J Mol Biol 12:600–611
15. Butler J, Jayson GG, Swallow AJ (1975) Biochim Biophys Acta 408:215–222
16. Dickerson RE, Timkovich R (1975) In: Boyer PD (ed) The enzymes XI, part A. Academic, New York, pp 397–547
17. George P, Tsou CL (1952) Biochem J 50:440–448
18. Stubbe J, Nocera DG, Yee CS, Chang MCY (2003) Chem Rev 103:2167–2202
19. Van Gelder BF, Slater EC (1962) Biochim Biophys Acta 587:593–595
20. Margalit R, Schejter A (1973) Eur J Biochem 32:492–499
21. Simic MG, Taub IA, Tocci J, Hurwitz PA (1975) Biochem Biophys Res Commun 62:161–167
22. Koppenol WH, van Buuren KJH, Butler J, Braams R (1976) Biochim Biophys Acta 449:157–168
23. Bielski BHJ (1978) Photochem Photobiol 28:645–649
24. Sawada Y, Yamazaki I (1973) Biochim Biophys Acta 327:257–265
25. Ivanovic-Burmazovic I, Filipovic MR (2012) Adv Inorg Chem 64:54–95
26. Bounds PL, Winston GW (1991) Free Radic Biol Med 11:447–453